63-2-6

UNCLASSIFIED

GRANTEE: WORLD LIFE RESEARCH INSTITUTE
22022 CENTER STREET
RECHE CANYON, COLTON
CALIFORNIA

GRANT NO: DA-CML-18-108-61-G-19

FINAL REPORT

Covering the Period

1 June 1961 - 30 September 1962

AN INVESTIGATION OF CIGUATERA POISON

Prepared by

Bruce W. Halstead, M.D., Donald W. Hessel Ph.D.

and John Suchy, Ph.D.

8 March 1963

Copy 9 of 20 Copies

UNCLASSIFIED

UNCLASSIFIED
1. Fish, Poisonous
2. Marine biotoxins
3. Grant No. DA-CML-18-108-61-G-19

AD_____ Accession No. _____

World Life Research Institute, Colton, California

AN INVESTIGATION OF CIGUATERA POISON

B. W. Halstead, D. Hessel, and J. Suchy

Final Progress Report, 8 March 1963, 11 pages
Grant No. DA-CML-18-108-61-G-19

During the period of this grant, 21 August through 20 September, an expedition was sent to Wake Island for the purpose of procuring poisonous fishes. Approximately 500 pounds of fishes were collected. The moray eels, Gymnothorax javanicus, proved to be strongly toxic.

Methanol extracts were prepared from a large series of red snapper, Lutjanus bohar, and moray eels, Gymnothorax javanicus. The partial purification procedure utilized precipitation of non-toxic material from methanol solution and adsorption of ciguatera toxin on silicic acid as previously developed by Hessel.

With the loss of Dr. Donald Hessel to the program, Dr. John Suchy continued the extraction program following a slightly modified procedure. Approximately fifty different fraction of varying degrees of purification and wight are presently frozen and in storage awaiting further processing.

It is believed that ciguatera poison has definite BW-CW significance, and that further research on the isolation and structure of the poison is indicated.

INTRODUCTION

This present grant is a continuation of the work that was formerly conducted under Contract No. DA18-108-405-CML-800 from the Army Chemical Corps.

Ciguatera is a disease caused by the ingestion of certain marine fishes that contain a neurotoxin of unknown nature and origin. Outbreaks of poisoning have been largely limited to a circumglobal belt from 35°N. to 34°S. latitude, with the Caribbean and tropical Pacific areas showing a relatively high incidence.

More than 300 species of tropical fishes have been incriminated as transvectors of this poison. The toxicity of a particular species varies widely from specimen to specimen, and different geographical areas may present radically different toxicity patterns. The fishes in one region may be highly toxic whereas those in adjacent areas may be completely inocuous. There is little evidence to suggest a distinct seasonal variation in toxicity of the ciguatera-producing fishes. Outbreaks of human poisonings may and have occurred during all seasons of the year.

The origin of the poison and the method of transmission are unknown. However, there is an increasing amount of evidence that the poison is derived from blue-green marine algae, and that one of the causative offenders is the species Lyngbya majuscula. Other species have also been suggested. At any rate, there appears to be a food-chain mechanism involved. Toxicity in fishes may develop rapidly - within a matter of several hours - as was observed in

the outbreaks which occurred in the Line Islands during 1943 through 1946.

The possibility of ciguatera poison being subject to manipulation or triggering, and its potential use as a biological-chemical warfare weapon system, has been repeatedly pointed up both in classified and unclassified documents, but to this date, has not been given serious consideration by this Government regarding this aspect of the problem.

Ciguatera is a debilitating type of intoxication characterized by a broad spectrum of neurotoxic manifestations. Severe gastro-intestinal upset with vomiting and diarrhea, tingling and numbness about the lips, tongue and throat, extreme weakness and exhaustion, visual disturbances, and a reversal of normal temperature sensations are often noted. One of the most characteristic symptoms is the extreme muscular debility that may prostrate an individual for an extended period of time. From the viewpoint of chemical and biological warfare, this intoxication has the advantage of a very low mortality rate (less than 7%), but the recovery rate is very prolonged - requiring months and sometimes years.

Serious researches on the chemistry of ciguatera poison are of recent origin - the bulk of the effort in the United States and abroad being conducted since 1956. Prior to the work under Contract No. DA18-108-405-CML-800, there was only one published report on the chemistry of ciguatera poison and that was by Hashimoto (1956, Bull. Japan. Soc. Sci. Fish., 21:1153). He found that the crude toxin was soluble in acetone and ether, but insoluble in water acidified with tartaric acid. The ether and acetone solubilities strongly suggested that the toxic agent was lipoid in nature and not the same as paralytic shellfish poison or tetrodotoxin (puffer poison).

Attempts to isolate ciguatera poison were started about 1957 by Banner, Helfrich, Burroughs and their associates, but the publications that have appeared to date reveal little about ciguatera toxin except for some solubility data, possible sensitivity of the toxin to oxygen, and details on the use of the mongoose as a test animal.

Past work by Halstead and his associates on the ciguatera problem was concerned largely with such activities as surveys of poisonous fishes, screening procedures, assay techniques, and methods of extraction and fractionation. Hessel, Halstead, and Peckham (1960, Ann. N. Y. Acad. Sci., 90(3):788) reported a procedure for removing the toxin in crude form from fish muscle. Acetone and ether were used in the extractions. Also described was a new bioassay method which is based upon the toxin's ability to attenuate the action potential of excised frog sciatic nerve. Based upon this assay, a study was conducted on the frequency of toxicity in the red snapper (Lutjanus bohar) from Palmyra, Line Islands, and also on the concentration of ciguatera poison in the musculature. It was found that increasing fish size is paralleled by a higher incidence of toxicity and a higher concentration of poison.

Under sponsorship of Contract No. DA18-405-CML-800, Hessel (1961, Tox. Appl. Pharmacol., 3:574) published a subsequent report on a procedure for the extraction and partial purification of ciguatera toxin from the muscle of the red snapper (Lutjanus bohar) by dissolving the toxin in warm methanol and precipitating nontoxic contaminants by cooling to -20°C. After recovery of the toxin from the methanol by evaporation of the solvent, subsequent fractionation was accomplished by silicic acid chromatography. Four broad

groups of substances were collected. Bioassays based on feeding experiments with cats, intraperitoneal injection of aqueous emulsions into white Swiss-Webster mice, and the reduction of action potential of excised frog sciatic nerve preparations, indicated that ciguatera toxin was carried on through the entire extraction-fractionation process to the last two groups of the silicic acid chromatographic separation. His results indicated that ciguatera may be caused by more than one neurotoxic substance. This published report essentially summarized the bulk of the work that was carried out under Contract No. DA18-108-405-CML-800 with the single exception of some unpublished research on Lyngbya majuscula, the blue-green algae which has been incriminated on several occasions as being associated with the origin of ciguatera poison in the food chain of the fish.

Samples of Lyngbya majuscula collected at Palmyra Island were subjected to a similar fractionation process using the silicic acid column. It was found that the toxic principle as shown by the frog nerve test was removed from silicic acid by ether. The two fractions corresponding to the ciguatera fractions from silicic acid were weakly toxic. If this was the same poison as found in fishes, it would indicate that the toxin concentration is low and apparently undergoes some concentration or storage in the body of the fishes feeding on the algae.

OBJECTIVES OF RESEARCH

The objectives of the research to be undertaken under the terms of this Grant No. DA-CML-18-108-61-G-19 were to:

a) Procure poisonous fishes at Palmyra Island, and,

b) Isolate and purify samples of the active principle of ciguatera poison in amounts required for structure determinations and activity screening, and for elucidation of the chemical nature and structure of the poison.

Procurement Program: During the period of 21 August through 20 September 1961, an expedition was conducted in the tropical Pacific for the purpose of procuring poisonous red snappers, Lutjanus bohar, and moray eels, Gymnothorax javanicus. It was originally intended that the expedition would be made to Palmyra Island, but because of the lack of logistics support and the limitation of finances, this was not possible. The next preferable site was Johnston Island, but due to highly classified operations that were being conducted at that time, this too had to be changed. Finally, Wake Island was selected with the hope that poisonous fishes could be obtained.

The scientific party consisted of the following:

Bruce W. Halstead, M.D., Project Director

Donald Hessel, Ph.D., Assistant Project Director

Richard Beltz, Ph.D., Biochemist

Don Ollis, Underwater Photographer

Robert Retherford, Photographer and Diver

Joy Halstead, Research Assistant

Marie Sachet, Ph.D., Botanist, National Research Council

The expedition was sponsored jointly by the ACC, the U. S. Coast Guard, and the U. S. Air Force.

Very little information is actually known regarding the incidence of poisonous fishes at Wake Island, although vague reports have appeared from time to time. Only one man has ever worked on the poisonous fishes of this Island and that is Dr. S. G. Ross, a British physician, who was stationed on the Island about 1950. Unfortunately, his findings were never reported in literature and little else is known regarding the subject.

Wake Island is a V-shaped atoll in the northwestern Pacific, north of the Marshall Islands between Midway and Guam. The atoll consists of three islets - Wake, Peale, and Wilkes. The total land area is about 2,185 sq. mi., and the highest point on the Island is about 12 feet (see Atoll Bull. No. 66, U. S. Nat. Res. Counc., for additional details of the structure of the Island). Wake is a fairly typical atoll having the usual array of corals and most of the general Indo-Pacific fish fauna.

The reefs on the leeward side of the Island on either side of the main boat channel between Wilkes and Wake Island are among the most spectacular of any that we have visited to date in any part of the world. Deep coral canyons dotted with living corals in abundance make this a beautiful place in which to collect. The water is a deep azure blue and during a period of calm is very clear with a visibility of from 100 feet or more. The water temperature ranges about the Island from 79.1° to 86°F. The warmest water period is said to be during August to September, but the period of best weather is about March through May.

Reef fishes are abundant, but somewhat singular in the distribution of certain species - for example, the usual array of red snappers was absent. We did not see any examples of Lutjanus bohar, or L. gibbus, and only a very few specimens of L. vaigiensis, and the few that were seen were very young. Strange to say, there was no evidence of older specimens of L. vaigiensis even in deep water areas where normally one would expect to find them. Moray eels were present, but nowhere were they abundant. Puffers were present in only modest numbers.

Visits were made to the windward side of the Island and to various parts of the lagoon, but none of these areas proved to be very productive. Data regarding the fish fauna of Wake Island is largely lacking and is very much needed.

Excellent collecting and deep freeze facilities are readily available at Wake Island.

A collection of puffers, porcupine fish, moray eels, surgeonfish, and an assortment of smaller reef fishes were collected. An estimated total of about 500 pounds of fishes were shipped back to California from Wake Island.

In general, this is not an ideal site for the collection of large numbers of poisonous fishes and does not remotely compare with the availability of poisonous fishes at Palmyra Island.

A total of about 1500 feet of underwater movie film and several hundred kodachromes were taken of the collecting activities.

Chemistry of Ciguatera Poison: The chemical work during the period from 1 June through September 1961, was conducted by Dr. Donald Hessel. All subsequent work was done by Dr. John Suchy.

The procedure followed during the initial period was the same as previously mentioned in this report under Contract No. DA-18-108-405-CML-800.

When the work was taken over by Dr. John Suchy, the procedure was modified slightly. The lengthy procedure of removing the water by placing the ground fish under vacuum was eliminated. Instead, the ground fish muscle was first placed into a 60°C oven until most of the water was removed by evaporation.

Each batch of the dried material representing about 500 grams of fresh fish or eel muscle was ground to a fine powder and extracted with 1000 ml. of acetone by first macerating the mixture at 50°C for fifteen minutes and then submitting it for a like period of vigorous agitation under the VirTis blender followed by a brief (5-minute) period of ultrasonification. The acetone extract was removed by suction filtration and the filter cake was treated two more times in the same manner with 400 ml. portions of acetone. The combined acetone filtrates were freed from solvent by evaporation under vacuum at 50°C.

The ether-methylene chloride purification procedure was the same as that used by Hessel. The consecutive quantities of ether used were approximately 100 ml., 60 ml. and 40 ml. Those of methylene chloride were 40 ml. and 20 ml. The combined ether-methylene chloride extractions were filtered and evaporated under vacuum and at 50°C until free from solvent. The yellow oily liquid thus obtained was treated with 200 ml. of acetone and chilled in the deep freeze until a white curd separated from the supernatant liquid. Following subsequent filtration, the residual contents of the flask and the precipitated material on the filter were treated twice with 50 ml. volumes of acetone. Following evaporation of the solvent under vacuum and at 50°C the oily liquid thus obtained was vigorously shaken with warm methanol (50°C) in the amount of approximately 100 ml. per gram of extract, ultrasonified and cooled in the deep freeze at temperatures varying between -10° to -20°C. A white curd

separated and settled at the bottom of the flask. After filtration and centrifuging at -20°C the rest of the procedure followed the course utilized by Hessel with some modification of the gradient elution equipment used in the final chromatographic separation.

The eluted fractions 5, 6 and 7 were freed from solvent, transferred to 5 ml. vials, stoppered and placed into storage in the deep freeze. About 50 different fractions of varying degress of processing and weight are presently in cold storage and awaiting final disposition. It was the original intention that work on these extracts would continue, but it appears at the moment that this will not be possible with the present lack of research support.

Unanticipated Delays: Upon the return of the Project Director, Dr. Bruce W. Halstead, from Wake Island, on 20 September 1961, it was found that a default in one of our major contracts had been incurred, placing the World Life Research Institute on the brink of bankruptcy. This situation was explained in detail in a letter to Mr. E. W. Bankert, Chief, R & D Procurement Division, U. S. Army Chemical Center, dated 13 November 1961. It was therefore requested that an extension of the grant time period be given without additional funds. This request was granted in the form of a four month's extension in a letter from Mr. E. W. Bankert dated 13 March (CMLMC-PA-2, DA-CML-18-108-61-G-19). It was the result of this situation that necessitated the replacement of Dr. Donald Hessel with Dr. John Suchy. Work has since progressed without any further complications.